THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 261, No. 14, Issue of May 15, pp. 6300-6306, 1986
Printed in U.S.A.

Ryanodine Activation and Inhibition of the Ca^{2+} Release Channel of Sarcoplasmic Reticulum*

(Received for publication, December 23, 1985)

Gerhard Meissner

From the Departments of Biochemistry and Nutrition, and Physiology, University of North Carolina, Chapel Hill, North Carolina 27514

The effect of the plant alkaloid ryanodine on the skeletal muscle sarcoplasmic reticulum Ca^{2+} release channel was studied by determining the Ca^{2+} permeability of "heavy" vesicles passively loaded with $^{45}Ca^{2+}$ in the presence or absence of ryanodine. Depending on the experimental conditions, ryanodine either stimulated or inhibited Ca^{2+} efflux. Vesicles were rendered permeable to $^{45}Ca^{2+}$ at a ryanodine concentration of 0.01 μM when diluted into a medium containing the two Ca^{2+} release channel inhibitors Mg^{2+} and ruthenium red. At ryanodine concentrations greater than 10 μM, $^{45}Ca^{2+}$ efflux was inhibited in channel-activating (5 μM Ca^{2+}) or -inhibiting (10 mM Mg^{2+} plus 10 μM ruthenium red) media. An optimal stimulatory effect was observed when vesicles were incubated with ryanodine at 37 °C and in media that caused partial opening of the channel. Similar results to those described above were obtained using cardiac sarcoplasmic reticulum vesicles that were capable of rapid $^{45}Ca^{2+}$ efflux.

Use of the slowly permeating molecule L-[^{3}H]glucose allowed measurement of channel-mediated efflux rates from vesicles in the presence and absence of ryanodine. At low activating concentrations, ryanodine did not appreciably change the regulation of L-glucose efflux rates by external Ca^{2+}, Mg^{2+}, and adenine nucleotide. These results suggested two possible modes of action of ryanodine: 1) a change in the gating mechanism of the channel which is not readily detected using the slowly permeating molecule L-glucose or 2) a change in channel structure which prevents its complete closing.

The sarcoplasmic reticulum (SR[1]) in muscle is a specialized intracellular membrane system which rapidly sequesters and releases Ca^{2+} (for reviews see Refs. 1–3). Sequestration of the released Ca^{2+} into SR is mediated by the Ca^{2+}-stimulated Mg^{2+}-dependent ATPase or Ca^{2+} pump. Release of Ca^{2+} from SR is triggered by an action potential originating at the neuromuscular junction and communicated to SR via an extension of the surface membrane, the T-tubule. Although the mechanism by which T-tubule depolarization induces Ca^{2+} release from SR has remained obscure, recent studies have indicated the presence of a ligand-gated SR "Ca^{2+} release channel" which is activated by Ca^{2+} and adenine nucleotides and inhibited by Mg^{2+} (4–13).

Ryanodine is a neutral plant alkaloid with profound effects on muscle (14). This naturally occurring product, as isolated from *Ryania speciosa*, is composed of two major active components, ryanodine and 9,21-didehydroryanodine, in a ratio of 1:3 (15, 16). The two components display equal toxicity and bind at nanomolar concentrations in a Ca^{2+}-dependent manner to heavy SR vesicle preparations from skeletal and cardiac muscle (17, 18).

As pointed out by Jenden and Fairhurst (14) and Sutko *et al.* (19), the effects of ryanodine on muscle are complex and depend on muscle type, calcium activity, pattern of muscle stimulation, as well as ryanodine concentration. For example, in vertebrate skeletal muscle, ryanodine produces an irreversible contraction, yet in cardiac muscle a decline in contractile force is observed. In isolated SR vesicle fractions, ryanodine has been assumed to activate or inhibit a Ca^{2+} permeable pathway. Cardiac SR contains a subpopulation of vesicles which exhibit an increased Ca^{2+}-loading activity and Ca^{2+} loading/ATP hydrolysis coupling ratio in the presence of ryanodine (20, 21). In a recent study, ryanodine initially induced a slight inhibition of oxalate-supported Ca^{2+} uptake followed by a later stimulation of Ca^{2+} uptake (22). In "heavy" skeletal muscle SR vesicle preparations, isolated by centrifugation between 2000 and 8000 × *g*, ryanodine increased the rate of Ca^{2+} efflux from vesicles which had been actively loaded with Ca^{2+} (23). A more recent study showed that high concentrations of ryanodine (300 μM) increased oxalate-supported Ca^{2+} uptake by junctional SR vesicle fractions, presumably by blocking a Ca^{2+}-permeable pathway (24). By contrast, at submicromolar concentrations, ryanodine appeared to lock the Ca^{2+} release channel of SR in an "open state" (18). Because most previous studies have utilized actively loaded vesicles, it is difficult to distinguish between the effects of ryanodine on Ca^{2+} uptake and/or efflux. In the present study, the permeability of the SR membrane to Ca^{2+} was more directly measured by using SR vesicles which were passively loaded with $^{45}Ca^{2+}$ in the absence of ATP. Evidence is presented which suggests that heavy SR vesicles contain a Ca^{2+} release channel which can be activated or inhibited by ryanodine, depending on the experimental conditions. A preliminary account of part of this work has appeared (25).

EXPERIMENTAL PROCEDURES

Materials—Ryanodine, originally obtained from the S. P. Penick Corp., was the generous gift of Drs. Larry Jones (Indiana University School of Medicine, Indianapolis, IN) and Joseph Feher (Medical College of Virginia, Richmond, VA). $^{45}Ca^{2+}$ was purchased from ICN Pharmaceuticals, Irvine, CA, and the ATP analog AMP-PCP from Sigma. All other reagents were of reagent grade.

Isolation of Membranes—Heavy rabbit skeletal muscle SR Ca^{2+}

* This research was supported by United States Public Health Service Grants AM 18687 and HL 27430. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] The abbreviations used are: SR, sarcoplasmic reticulum; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; AMP-PCP, adenosine 5′-(β,γ-methylenetriphosphate); Pipes, 1,4-piperazinediethanesulfonic acid.

release vesicle fractions were recovered from the 36–45% region of a sucrose gradient that contained membranes sedimenting at 2,600–35,000 × *g* (13). The "control" vesicle fraction which lacks the Ca^{2+} release channel was recovered from the 30–34% region of a sucrose gradient containing membranes obtained by differential pelleting at 35,000–130,000 × *g*.

Cardiac muscle SR vesicles were isolated from approximately 100 g of canine ventricular tissue. Muscle (50 g) was minced and homogenized in 350 ml of 0.3 M sucrose, 1 mM diisopropyl fluorophosphate, a protease inhibitor, and 20 mM K Pipes, pH 7.4, at 4 °C for 60 s in a Waring blender. The homogenate was centrifuged for 20 min at 8,000 rpm (10,500 × *g*) in a GSA rotor in a Sorvall RC-2 centrifuge. A crude microsomal fraction was obtained from the supernatant by centrifugation for 60 min at 33,000 rpm (90,000 × *g*) in a Beckman type 35 rotor. The pellets were resuspended in 0.3 M sucrose containing 0.4 M KCl, 0.1 mM $MgCl_2$, 100 μM EGTA, 100 μM Ca^{2+}, and 5 mM K Pipes, pH 6.8, and placed in a Beckman Ti 14 zonal rotor on top of a linear 20–45% (w/w) sucrose gradient containing 0.4 M KCl, 0.1 mM $MgCl_2$, 100 μM EGTA, 100 μM Ca^{2+}, and 5 mM K Pipes, pH 6.8. After centrifugation for 180 min at 40,000 rpm, membranes sedimenting between 19–25%, 25–28%, 28–31%, 31–33%, and 33–40% sucrose (as measured with a refractometer at 22 °C) were collected, diluted with 1.5 volumes of 0.4 M KCl, and sedimented by centrifugation for 60 min at 33,000 rpm in a Beckman type 35 rotor. The pellets were resuspended in 0.3 M sucrose and 5 mM K Pipes, pH 6.8, quickly frozen, and stored at −135 °C. Among the five gradient fractions, the 31–40% sucrose gradient fractions displayed the highest Ca^{2+} release activity. Between 40–60% of the $^{45}Ca^{2+}$ retained by the vesicles in a medium containing 10 mM Mg^{2+} and 10 μM ruthenium was rapidly released on dilution into a medium containing 5 μM free Ca^{2+} (*cf.* Fig. 6).

$^{45}Ca^{2+}$ Flux Measurements with Passively Loaded Vesicles—$^{45}Ca^{2+}$ efflux rates from vesicles passively loaded with $^{45}Ca^{2+}$ were determined by Millipore filtration, as previously described (10, 13). Unless otherwise indicated, vesicles (5–10 mg of protein/ml) were incubated at 22 °C in a loading medium containing 20 mM K Pipes, pH 7, 0.1 M KCl, 0.1 mM EGTA, and 0.2–5.1 mM $^{45}Ca^{2+}$. After 2 h, samples received an addition of 0.1 volume of loading medium containing the dissolved ryanodine, followed by incubation for additional times at 22 and 37 °C. $^{45}Ca^{2+}$ efflux behavior of the vesicles was assessed by diluting vesicles into iso-osmolal unlabeled Ca^{2+} release channel-inhibiting (10 mM Mg^{2+} plus 10 μM ruthenium red) or Ca^{2+} release channel-activating (1 mM EGTA plus 0.93 mM Ca^{2+}; 5 μM free Ca^{2+}) media. Untrapped as well as released $^{45}Ca^{2+}$ was separated away by placing the vesicles on 0.45-μm HAWP Millipore filters followed by rapid rinsing to remove extravesicular $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ radioactivity retained by the vesicles on the filters was determined by liquid scintillation counting.

$^{45}Ca^{2+}$ flux measurements were carried out at least in duplicate with two or more time points. For a given preparation the standard errors were ±10% or less.

Biochemical Assays—Protein was determined by the Lowry method using bovine serum albumin as a standard. Free Ca^{2+} concentrations were adjusted using the Ca^{2+}-chelating agent EGTA and were calculated according to a computer program using binding constants published by Fabiato (26).

RESULTS

Measurement of $^{45}Ca^{2+}$ Release from Passively Loaded Vesicles—The presence of a Ca^{2+} release channel that could mediate the rapid release of intravesicular Ca^{2+} stores is shown in Fig. 1. Heavy SR Ca^{2+} release vesicles were passively loaded with 1 mM $^{45}Ca^{2+}$ and then either diluted into a medium which inhibited or activated the Ca^{2+} release channel of SR. $^{45}Ca^{2+}$ efflux was slow in Ca^{2+} release channel-inhibiting medium which contained 10 mM Mg^{2+} and 10 μM ruthenium red. This allowed determination of the amount of $^{45}Ca^{2+}$ (44 nmol/mg of protein) that was trapped by all intact vesicles (Fig. 1*A*). In contrast, the vesicles released most of the $^{45}Ca^{2+}$ within 30 s when diluted into Ca^{2+} release channel-activating medium which contained 5 μM free Ca^{2+}. With the use of a rapid quench apparatus (13) it could be shown that $^{45}Ca^{2+}$ was released with a first order rate constant of about 1 s^{-1} (Fig. 1*B*). The small amount of $^{45}Ca^{2+}$ (6 nmol/mg of protein) that remained within the vesicles 30–60 s after dilution into the 5 μM Ca^{2+} medium reflected a subpopulation of vesicles that lacked the Ca^{2+} release channel (10).

The Effect of Ryanodine on $^{45}Ca^{2+}$ Release—The effect of ryanodine on the Ca^{2+} release behavior of heavy SR vesicles was determined by preincubating vesicles with or without ryanodine and by diluting them into media containing or lacking ryanodine. Dilution into Ca^{2+} release channel-inhibiting media containing 0, 1, or 150 μM ryanodine gave similarly slow $^{45}Ca^{2+}$ efflux rates (not shown). $^{45}Ca^{2+}$ efflux was also not appreciably affected when vesicles not pretreated with ryanodine were diluted into 5 μM Ca^{2+} release media containing 1 μM ryanodine (Fig. 1*B*) while dilution of the vesicles into a release medium containing 150 μM ryanodine increased the $^{45}Ca^{2+}$ efflux rate about 1.5-fold.

Prolonged incubation of the vesicles with ryanodine drastically altered their release behavior. Incubation with 1 μM ryanodine for 20 min at 37 °C enhanced the amount of $^{45}Ca^{2+}$ that was released by vesicles in the Ca^{2+} release channel-inhibiting medium (Fig. 1*A*). Incubation with a high concentration of ryanodine (150 μM) had the opposite effect. In this case, only a small fraction of the vesicle $^{45}Ca^{2+}$ was released in channel-activating or -inhibiting media. Most of the trapped $^{45}Ca^{2+}$ was present in a releasable form since less than 1 nmol of $^{45}Ca^{2+}$/mg of protein was retained by the vesicles when the Ca^{2+} ionophore A23187 (2 μg/ml) was added to the release media.

Control experiments showed that ryanodine did not appreciably alter the permeability of the Ca^{2+} release vesicles to [^{14}C]sucrose. Further, it was established that ryanodine did not change the Ca^{2+} permeability of control SR vesicles which lack the Ca^{2+} release channel. These results suggested a specific interaction of ryanodine with the Ca^{2+} release channel or a related structure in heavy SR vesicles.

The time course of stimulation and inhibition of Ca^{2+} release by ryanodine was determined by preincubating heavy SR vesicles for varying times at 22 and 37 °C with 1 or 100 μM ryanodine (Fig. 2*A*). Incubation with 1 μM ryanodine at 22 °C in the presence of 1 mM Ca^{2+} caused the gradual release of $^{45}Ca^{2+}$ in the Ca^{2+} release channel-inhibiting medium. Channel "opening," *i.e.* loss of $^{45}Ca^{2+}$ from heavy SR vesicles, could be accelerated by either raising the incubation temperature to 37 °C or by increasing the ryanodine concentration to 100 μM. Prolonged incubation with 100 μM ryanodine at 22 °C appeared to cause a gradual "closing" of the channel in channel inhibiting and activating media. This closing process was accelerated at 37 °C.

The effectiveness of ryanodine in stimulating and inhibiting $^{45}Ca^{2+}$ release from heavy SR vesicles was dependent on the composition of the vesicle incubation media. Addition of 5 mM Mg^{2+} slowed the time course of ryanodine activation and inhibition of $^{45}Ca^{2+}$ release (Fig. 2*B*). Other conditions that retarded $^{45}Ca^{2+}$ release activation and inhibition were the addition of 10 μM ruthenium red or an increase in Ca^{2+} concentration to 10 mM, while lower Ca^{2+} concentrations rendered the vesicles more sensitive to ryanodine. At 0.1 mM $^{45}Ca^{2+}$, rapid $^{45}Ca^{2+}$ release occurred from most Ca^{2+} release vesicles after incubation with 0.01 μM ryanodine for 45 min at 37 °C (Fig. 3). Incubation with 100 μM ryanodine rendered most vesicles impermeable to $^{45}Ca^{2+}$ in Ca^{2+} release channel-activating and -inhibiting media. Taken together, data of Figs. 2 and 3 suggest that stimulation or inhibition of Ca^{2+} efflux was dependent on ryanodine concentration, temperature, and time of incubation, as well as external divalent cation concentration.

A change in free Ca^{2+} or nucleotide concentration greatly

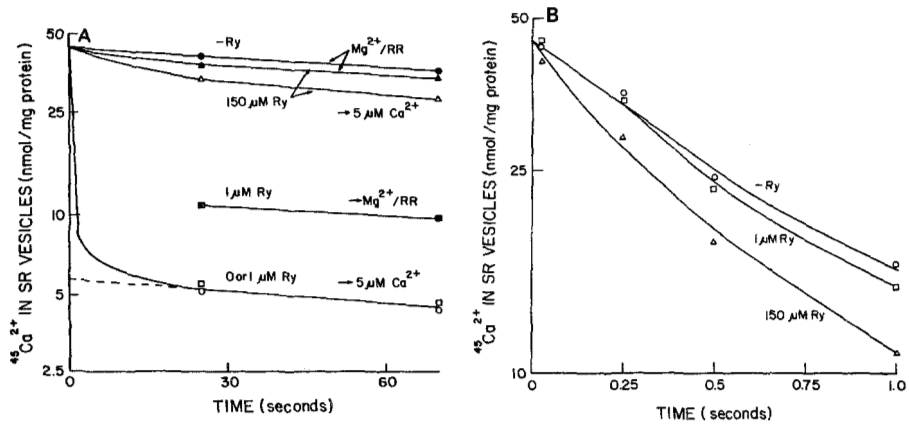

FIG. 1. **Effect of ryanodine on $^{45}Ca^{2+}$ efflux from skeletal muscle SR Ca^{2+} release vesicles.** *A*, a heavy SR Ca^{2+} release vesicle fraction (5 mg of protein/ml) was incubated for 2 h at 22 °C and 20 min at 37 °C with 1 mM $^{45}Ca^{2+}$ in the presence of 0, 1, or 150 μM ryanodine (*Ry*). $^{45}Ca^{2+}$ efflux was initiated by diluting vesicles 20-fold into release media containing either 10 mM Mg^{2+} plus 10 μM ruthenium red (*RR*, ●, ▲, ■) or 1 mM EGTA plus 0.88 mM Ca^{2+} (5 μM free Ca^{2+} after the addition of the vesicles) (○, △, □). $^{45}Ca^{2+}$ efflux was terminated by placing vesicles on 0.45-μm Millipore filters followed by rapid rinsing with release medium to remove extravesicular $^{45}Ca^{2+}$. Amounts of $^{45}Ca^{2+}$ trapped by all intact vesicles in the incubation medium as well as amounts not readily released in the 5 μM Ca^{2+} release medium were obtained by back extrapolation to the time of vesicle dilution. For vesicles not incubated with ryanodine, the amounts corresponded to 44 and 6 nmol/mg protein, respectively. *B*, an Update System 1000 chemical quench apparatus was used to determine the initial $^{45}Ca^{2+}$ efflux rates in the 5 μM free Ca^{2+} release medium (13). Vesicles (2 mg of protein/ml) were incubated for 2 h at 22 °C with 1 mM $^{45}Ca^{2+}$. $^{45}Ca^{2+}$ efflux was initiated by diluting vesicles with 4 volumes of release medium containing 6.25 mM EGTA, 5.55 mM Ca^{2+}, and 0 (○), 1.25 (□), or 187.5 μM (△) ryanodine. Rapid $^{45}Ca^{2+}$ efflux was inhibited at the indicated times by the addition of 4 additional volumes of a quench solution containing 22.5 mM Mg^{2+} and 22.5 μM ruthenium red. Vesicles were subsequently placed on 0.45-μm Millipore filters and rinsed with a medium containing 10 mM Mg^{2+} and 10 μM ruthenium red.

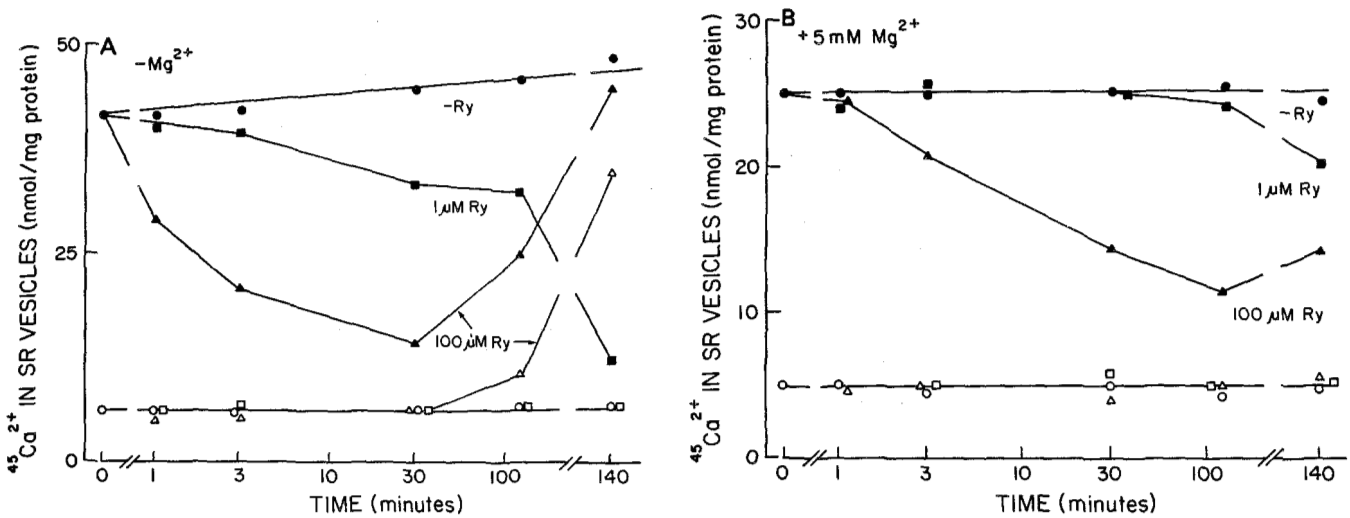

FIG. 2. **Time course of stimulation and inhibition of $^{45}Ca^{2+}$ release by ryanodine.** A skeletal muscle SR Ca^{2+} release fraction (5 mg of protein/ml) was initially passively loaded with 1 mM $^{45}Ca^{2+}$ (*A*) or 1 mM $^{45}Ca^{2+}$ plus 5 mM Mg^{2+} (*B*) by incubation for 120 min at 22 °C. Vesicles were then incubated for the indicated times at 22 °C in the absence (●, ○) or presence of 1 (■, □) or 100 μM (▲, △) ryanodine (*Ry*). The 140-min time point was obtained by incubating vesicles in the presence or absence of ryanodine for 120 min at 22 °C and 20 min at 37 °C. $^{45}Ca^{2+}$ release was initiated by diluting vesicles 20-fold into release media containing either 10 mM Mg^{2+} plus 10 μM ruthenium red (●, ■, ▲) or 5 mM EGTA plus 4.6 mM Ca^{2+} (○, □, △). Amounts of $^{45}Ca^{2+}$ retained by the vesicles were obtained by back extrapolation to the time of vesicle dilution (*cf.* Fig. 1*A*).

affects the Ca^{2+} efflux rate from heavy SR vesicles (11, 13). The SR Ca^{2+} release channel rarely opens in the absence of external Ca^{2+} and nucleotide. Micromolar concentrations of Ca^{2+} or millimolar concentrations of ATP by themselves partially activate the channel by opening it for intermittent times. The presence of both Ca^{2+} and ATP maximally stimulates Ca^{2+} release, and the channel is open essentially all the time. Millimolar Ca^{2+} in the absence of nucleotide nearly fully inhibits the channel. In Fig. 4, vesicles were incubated at 37 °C in the presence or absence of 5 μM ryanodine in media which partially opened (5×10^{-6} M Ca^{2+} or 10^{-8} M Ca^{2+} plus 1 mM AMP-PCP) or nearly closed (10^{-8} M Ca^{2+} or 10^{-3} M Ca^{2+}) the Ca^{2+} release channel of SR. $^{45}Ca^{2+}$ influx was subsequently initiated at 23 °C in a 1 mM $^{45}Ca^{2+}$ medium and was stopped by the addition of 10 mM Mg^{2+} and 10 μM ruthenium red. The amount of $^{45}Ca^{2+}$ trapped by the vesicles was deter-

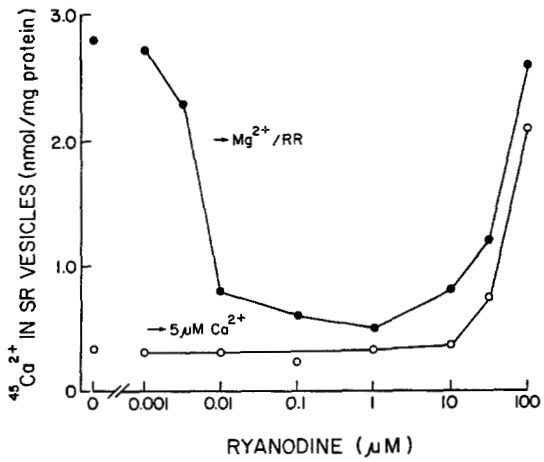

FIG. 3. **Dependence of $^{45}Ca^{2+}$ efflux on ryanodine concentration.** A skeletal muscle SR Ca^{2+} release vesicle fraction (5 mg of protein/ml) was incubated for 45 min at 37 °C with 0.1 mM free $^{45}Ca^{2+}$ and the indicated concentrations of ryanodine. Amounts of $^{45}Ca^{2+}$ retained by the vesicles in the 10 mM Mg^{2+} 10 μM ruthenium red (*RR*, ●) or 5 μM free Ca^{2+} (○) release media were determined as indicated in Fig. 1*A*.

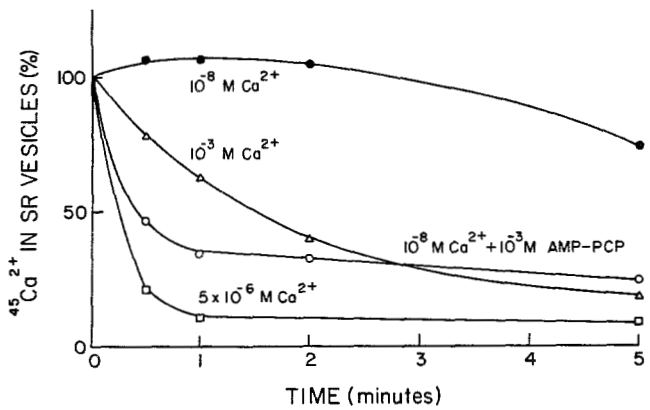

FIG. 4. **Effects of ryanodine on the Ca^{2+} permeability of skeletal muscle SR Ca^{2+} release vesicles incubated in the presence and absence of Ca^{2+} and AMP-PCP.** Skeletal muscle SR Ca^{2+} release vesicles (5 mg of protein/ml) were incubated in the presence of 5 μM ryanodine at 37 °C for the indicated times. Incubation media contained the indicated concentrations of free Ca^{2+} and AMP-PCP. Vesicles were subsequently diluted with 9 volumes of $^{45}Ca^{2+}$ influx medium yielding a $^{45}Ca^{2+}$ concentration of 1 mM and AMP-PCP concentration of 0.25 mM. $^{45}Ca^{2+}$ influx was stopped at 15 s by the addition of 25 volumes of a medium containing 10 mM Mg^{2+} and 10 μM ruthenium red. Vesicles were placed on 0.45-μm Millipore filters and rinsed with Ca^{2+} release channel-inhibiting medium. Data are expressed as the percentage of $^{45}Ca^{2+}$ that was retained by vesicles incubated in the absence of ryanodine.

mined by Millipore filtration. Vesicles not preincubated with ryanodine accumulated about 20 and 40 nmol/mg of protein when incubated with 1 mM $^{45}Ca^{2+}$ for 15 s and 10 min, respectively. Preincubation of the vesicles with ryanodine reduced the amounts of $^{45}Ca^{2+}$ retained by the vesicles on the Millipore filters (Fig. 4). A reduction in vesicle $^{45}Ca^{2+}$ suggested that, as observed in the $^{45}Ca^{2+}$ efflux experiments, Mg^{2+} and ruthenium red were unable to block the release of accumulated $^{45}Ca^{2+}$ on a time scale of 30–60 s. Ryanodine acted most rapidly in the two media containing either 5×10^{-6} or 10^{-8} Ca^{2+} plus 1 mM AMP-PCP. Thus, it appeared that an opening of the Ca^{2+} release channel favored its modification by ryanodine.

Table I shows that ryanodine stimulated and inhibited the

TABLE I

Effects of ryanodine on $^{45}Ca^{+}$ release from skeletal muscle SR vesicles

Skeletal muscle SR Ca^{2+} release vesicles were incubated with 1 mM $^{45}Ca^{2+}$ for 90 min at 22 °C. After the addition of 0 or 150 μM ryanodine, incubation was continued for 30 min at 22 °C or 30 min at 22 °C plus 30 min at 37 °C. Release media contained after addition of the vesicles the indicated concentrations of free Ca^{2+}, Mg^{2+}, nucleotide, and ruthenium red (RR). The amounts of $^{45}Ca^{2+}$ remaining with the vesicles at 30 s after dilution were determined by Millipore filtration. Vesicles retained 42 nmol of $^{45}Ca^{2+}$/mg of protein when incubated for 120 min at 22 °C in the absence of ryanodine and diluted into the 2×10^{-5} M Ca^{2+}, 10 mM Mg^{2+}, 10 μM RR release medium. Data were normalized by setting this amount equal to 100%.

Composition of release medium	$^{45}Ca^{2+}$ in SR vesicles			
	30 min at 22 °C		30 min at 22 °C plus 30 min at 37 °C	
	−Ryanodine	+Ryanodine	−Ryanodine	+Ryanodine
	%			
2×10^{-5} M Ca^{2+}, 10 mM Mg^{2+}, 10 μM RR	100	30	90	100
10^{-8} M Ca^{2+}	70	30	70	100
10^{-8} M Ca^{2+}, 5 mM ATP	15	15	15	85
10^{-8} M Ca^{2+}, 10 mM Mg^{2+}, 10 μM RR	97	38	90	100
5×10^{-6} M Ca^{2+}	15	15	15	95
5×10^{-6} M Ca^{2+}, 2.5 mM AMP-PCP	13	14	13	87
5×10^{-6} M Ca^{2+}, 10 mM caffeine			13	88
5×10^{-6} M Ca^{2+}, 1 mM Mg^{2+}	70	28	65	92
10^{-2} M Ca^{2+}	98	32		
10^{-4} M La^{3+}	115	35		

release of $^{45}Ca^{2+}$ under a variety of release conditions. In the absence of ryanodine and at 10^{-8} M Ca^{2+}, $^{45}Ca^{2+}$ efflux was relatively slow. About one-third of the $^{45}Ca^{2+}$ stores in the Ca^{2+} permeable vesicle population were released within 30 s, *i.e.* when the first time point was taken using the Millipore filtration technique. Increase in free Ca^{2+} to 4 μM or the addition of 5 mM ATP or the addition of the nonhydrolyzable ATP analog AMP-PCP to the 5 μM Ca^{2+} release medium yielded rate constants of $^{45}Ca^{2+}$ release ranging from about 1–100 s^{-1} (13). Thus, under these conditions the Ca^{2+} permeable vesicle population completely released its $^{45}Ca^{2+}$ stores within 30 s. $^{45}Ca^{2+}$ release was partially inhibited by adding 1 mM Mg^{2+} to the 5 μM Ca^{2+} release medium and fully inhibited by 10 mM Ca^{2+} or 0.1 mM La^{3+}. Prior exposure of the vesicles to 150 μM ryanodine for 30 min at 22 °C resulted in the rapid release of $^{45}Ca^{2+}$ in all release media. Specifically, ryanodine accelerated the release of vesicular $^{45}Ca^{2+}$ at 10^{-8} M. As observed in media containing Ca^{2+} in micromolar activating concentrations, Mg^{2+} and ruthenium red were unable to block $^{45}Ca^{2+}$ release at 10^{-8} M Ca^{2+} on a time scale of 30 s. Two other inhibitors of the channel that were rendered ineffective by ryanodine were La^{3+} and high Ca^{2+}.

Continued incubation of the vesicles with 150 μM ryanodine for 30 min at 37 °C inhibited the release of $^{45}Ca^{2+}$ from about 85% of the vesicles in all release media including those containing 5 μM Ca and the Ca^{2+}-releasing drug caffeine (1–3) or AMP-PCP (Table I). Under nearly optimally activating conditions (5 μM free Ca^{2+} and 2.5 mM AMP-PCP), the first order rate constant of $^{45}Ca^{2+}$ release was reduced from 45 s^{-1} to less than 0.01 s^{-1} (not shown).

Effect of Ryanodine on L-[^{3}H]Glucose Efflux—Both $^{45}Ca^{2+}$ efflux and L-[^{3}H]glucose efflux from heavy SR vesicles were similarly influenced by the composition of the release media. Vesicles slowly released their L-[^{3}H]glucose contents when diluted into 10 mM Mg^{2+} or 10^{-8} M free Ca^{2+} media (Fig. 5). About half of the trapped L-[^{3}H]glucose was released within 30 s when vesicles were diluted into a medium containing 5 μM free Ca^{2+} and 2.5 mM AMP-PCP. Rapid L-[^{3}H]glucose efflux, like that of $^{45}Ca^{2+}$, could be blocked by the addition of 10 mM Mg^{2+} or 10 μM ruthenium red (not shown). Measurement of the initial release rate using the two blocking agents indicated that in the presence of 5 μM free Ca^{2+} and 2.5 mM AMP-PCP, L-glucose-permeable vesicles released L-[^{3}H]glucose with a first order rate constant of about 25 min^{-1}. Omission of 2.5 mM AMP-PCP from the 5 μM Ca^{2+} release medium reduced the first order rate constant to about 3 min^{-1}. Under comparable conditions, the respective rate constants of $^{45}Ca^{2+}$ release were 45 and 1.2 s^{-1} (not shown), *i.e.* L-[^{3}H] glucose was released about 50 times slower than $^{45}Ca^{2+}$. The glucose permeability of light vesicles which lack the Ca^{2+} release channel was not appreciably changed under the experimental conditions of Fig. 5. It appeared, therefore, that L-glucose was capable of passing, albeit slowly, through the Ca^{2+}- and nucleotide-activated Ca^{2+} release channel.

Fig. 5 also shows the L-[^{3}H]glucose efflux behavior of heavy SR vesicles which were preincubated with ryanodine, using conditions (20 μM ryanodine for 120 min at 22 °C and 1 mM Ca^{2+}) that rendered about 75% of the Ca^{2+} release vesicles permeable to $^{45}Ca^{2+}$ in the Ca^{2+} release channel-inhibiting medium (not shown). In contrast to $^{45}Ca^{2+}$, L-[^{3}H]glucose efflux from ryanodine-treated vesicles was fairly slow in media containing 10 mM Mg^{2+} (Fig. 5) or 10 μM ruthenium red (not shown). It was, therefore, possible to determine the initial L-[^{3}H]glucose efflux rates by Millipore filtration. Ryanodine had a moderate effect on the L-glucose efflux behavior of the vesicles. The efflux rate from L-glucose-permeable vesicles was increased by a factor of about 2 in media containing 10^{-8} or 5×10^{-6} M free Ca^{2+} (Fig. 5). On the other hand, ryanodine decreased the rate constant of L-glucose efflux from about 25 to 10 min^{-1} when the Ca^{2+} release channel was nearly fully activated by diluting the vesicles into the Ca^{2+}- and nucleotide-containing medium. L-[^{3}H]Glucose efflux experiments suggest that at low concentrations of ryanodine the channel was not locked into a fully open configuration.

Incubation of the vesicles with 100 μM ryanodine under the conditions of Fig. 3 rendered most of the vesicles impermeable to L-[^{3}H]glucose in media containing 10 mM Mg^{2+} plus 10 μM ruthenium red or 5 μM Ca^{2+} plus 2.5 mM AMP-PCP.

Effect of Ryanodine on $^{45}Ca^{2+}$ Release from Cardiac Muscle SR Vesicles—In Fig. 6, cardiac muscle SR vesicles were passively loaded with 5 mM $^{45}Ca^{2+}$ and diluted into media containing either 10 mM Mg^{2+} plus 10 μM ruthenium red or 5 μM free Ca^{2+}. The vesicles retained 25 nmol of $^{45}Ca^{2+}$/mg of protein when diluted into the Ca^{2+} release-inhibiting medium. About 60% of this amount was released within 30 s in the 5 μM Ca^{2+} release medium. Thus, as in skeletal muscle (Fig. 1*A*), a subpopulation of cardiac muscle SR vesicles was capable of rapid $^{45}Ca^{2+}$ release.

Incubation of the vesicles with 1 μM ryanodine at 22 and 37 °C caused the rapid release of $^{45}Ca^{2+}$ from about half of the Ca^{2+} permeable vesicles in the Ca^{2+} release channel-inhibiting medium. At 100 μM, ryanodine acted on most cardiac Ca^{2+} release vesicles after an incubation period of only 2 min at 22 °C. Continued incubation with 100 μM ryanodine at 22 and 37 °C appeared to "close" the channel in channel-inhibiting and -activating media.

In contrast, the Ca^{2+} release behavior of skeletal muscle vesicles was not appreciably affected by incubation with 1 μM ryanodine in the presence of 5 mM Ca^{2+} (not shown). Also, as observed in the presence of 5 mM Mg^{+} (Fig. 2*B*), prolonged incubation at 22 °C with 100 μM ryanodine was required to render skeletal muscle Ca^{2+} release vesicles permeable to $^{45}Ca^{2+}$ in the channel-inhibiting medium. $^{45}Ca^{2+}$ release from skeletal muscle release vesicles was not appreciably inhibited by 100 μM ryanodine under the experimental conditions of Fig. 6. Thus, while ryanodine appeared to have a similar

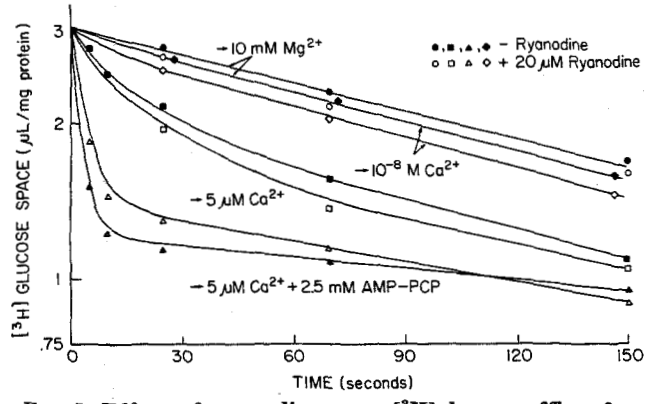

FIG. 5. **Effect of ryanodine on L-[^{3}H]glucose efflux from skeletal muscle SR Ca^{2+} release vesicles.** A SR Ca^{2+} release vesicle fraction (10 mg of protein/ml) was incubated for 2 h at 22 °C in a medium containing 20 mM K Pipes, pH 7.0, 0.1 M KCl, 1 mM L-[^{3}H]glucose, 0.1 mM EGTA, 1.1 mM Ca^{2+}, and either 0 (●, ■, ▲, ◆) or 20 μM ryanodine (○, □, △, ◇). L-[^{3}H]Glucose efflux was initiated by diluting vesicles 50-fold into iso-osmolal release media containing 1 mM L-glucose and either 10 mM Mg^{2+} (●, ○), 10^{-8} M free Ca^{2+} (◆, ◇), 5×10^{-6} M free Ca^{2+} (■, □), or 5×10^{-6} M free Ca^{2+} plus 2.5 mM AMP-PCP (▲, △). The time course of L-[^{3}H]glucose efflux was obtained by measuring the amounts of L-[^{3}H]glucose that remained with the vesicles at times ranging from 5 to 150 s. Five- and 10-s time points were determined by inhibiting L-[^{3}H]glucose efflux by the addition of 10 mM Mg^{2+} plus 10 μM ruthenium red.

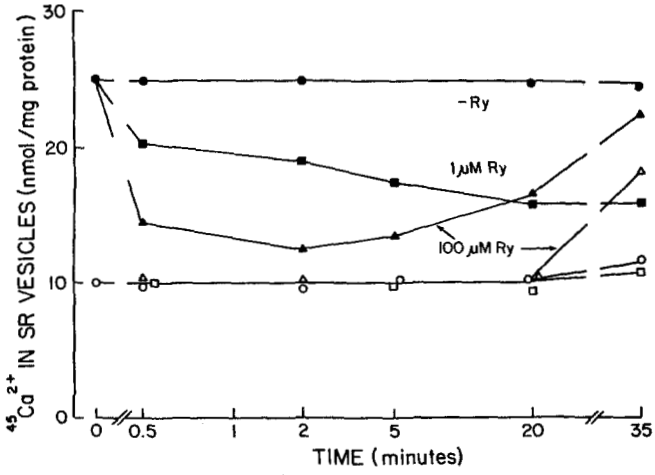

FIG. 6. **Effects of ryanodine on $^{45}Ca^{2+}$ efflux from cardiac muscle SR vesicles.** A cardiac muscle SR vesicle fraction (5 mg of protein/ml) sedimenting at 31–33% sucrose (*cf.* "Experimental Procedures") was passively loaded with 5 mM $^{45}Ca^{2+}$ by incubation for 60 min at 22 °C. Vesicles were then incubated for the indicated times at 22 °C with 0 (●, ○), 1 (■, □), or 100 μM (▲, △) ryanodine. The 35-min time point was obtained by incubating vesicles in the presence or absence of ryanodine for 20 min at 22 °C and 15 min at 37 °C. $^{45}Ca^{2+}$ release was initiated by diluting vesicles into release media containing 10 mM Mg^{2+} plus 10 μM ruthenium red (●, ■, ▲) or 5 μM free Ca^{2+} (○, □, △). Amounts of $^{45}Ca^{2+}$ retained by the vesicles were determined as indicated in Fig. 1*A*.

overall effect on the Ca^{2+} release behavior of cardiac and skeletal muscle SR vesicles, in the presence of 5 mM Ca^{2+}, ryanodine acted more rapidly on cardiac than skeletal muscle SR Ca^{2+} release vesicles.

DISCUSSION

A major aim of the present study was to define conditions that would allow ryanodine to "open" and "close" the Ca^{2+} release channel of SR. Stimulation or inhibition of Ca^{2+} efflux was influenced by the divalent cation concentration, temperature, and time of incubation, plus the concentration of ryanodine. At 37 °C and 0.1 mM Ca^{2+}, essentially all skeletal muscle SR Ca^{2+} release vesicles were rendered permeable to $^{45}Ca^{2+}$ at a ryanodine concentration of 0.01 μM when diluted into a medium containing the two Ca^{2+} release channel inhibitors Mg^{2+} and ruthenium red. Inhibition of $^{45}Ca^{2+}$ efflux was observed only at ryanodine concentrations in excess of 10 μM.

The apparent discrepancies in previous studies (18–24), where ryanodine stimulated or inhibited ATP-mediated Ca^{2+} uptake by skeletal and cardiac SR, can be resolved by considering the difference in assay conditions and the use of skeletal *versus* cardiac SR vesicles. Some of the criteria of ryanodine action noted above have been previously recognized. Stimulation of $^{45}Ca^{2+}$ efflux by ryanodine from actively loaded heavy or junctional terminal cisternae skeletal muscle SR vesicles was blocked by 5 mM Mg^{2+} (23) and ruthenium red (18). The effects of ryanodine on actively loaded cardiac vesicles were dependent on ryanodine concentration and temperature (22). Pessah *et al.* (17) observed that specific [^{3}H]ryanodine binding was optimal at 70 μM Ca^{2+} for heavy SR vesicle preparations from skeletal muscle and at 0.1–2 mM Ca^{2+} for cardiac SR preparations. Lower and higher Ca^{2+} concentrations as well as ruthenium red were inhibitory in both preparations, whereas Mg^{2+}, Ba^{2+}, Cd^{2+}, and La^{3+} inhibited [^{3}H]ryanodine binding to the skeletal preparation only.

[^{3}H]Ryanodine binding studies have shown that heavy skeletal muscle SR preparations specifically bind about 10 pmol of ryanodine/mg of protein with a K_D of 20–200 nM (17, 18). Cardiac preparations yielded nonlinear Scatchard plots indicating the presence of multiple receptor sites (0.5 pmol/mg of protein, K_D = 36 nM; and 1.7 pmol/mg protein, K_D = 340 nM; Ref. 17). Comparison of the binding data with the dose-response curves of Fig. 3 suggests that ryanodine stimulated the release of Ca^{2+} from heavy skeletal muscle SR vesicles by binding to high affinity sites of the Ca^{2+} channel.

The effectiveness of ryanodine appeared to be dependent on whether the Ca^{2+} release channel was present in an open or closed configuration. Preincubation with micromolar Ca^{2+} or 1 mM AMP-PCP in the presence of 10^{-8} M Ca^{2+} partially opened the channel and resulted in rapid modification of the channel by ryanodine (Fig. 4). Ryanodine acted less readily at 10^{-8} or 10^{-3} M Ca^{2+}, millimolar Mg^{2+}, or micromolar ruthenium red, *i.e.* when $^{45}Ca^{2+}$ release was slow. One possible explanation of these data is that ryanodine binds to a site which is accessible only in the open channel. Alternatively, ryanodine might first diffuse across the membrane before it binds to a site located on the inner surface of the vesicles. Diffusion of ryanodine may be facilitated by the open channel since relatively large molecules such as L-glucose can traverse the open channel.

The molecular basis for the inhibitory action of ryanodine is unclear. Using ryanodine concentrations of 20–500 μM, Feher and Lipford (22) have suggested that the binding of ryanodine to its cardiac receptor results in temporary activation of a Ca^{2+} channel which then slowly closes as it reaches a more stable configuration. An alternative explanation is the presence of a minor inhibitory component in the ryanodine extracts isolated from *Ryania speciosa*. In support of this suggestion, inactivation of Ca^{2+} release was observed only when ryanodine concentrations exceeded 10 μM (Fig. 3).

The Ca^{2+} flux measurements of previous studies have not clarified to what extent the SR membrane Ca^{2+} permeability is changed by ryanodine. One reason was that most previous studies used the Ca^{2+}-precipitating agent oxalate to increase the amounts of Ca^{2+} sequestered by the vesicles. Changes in Ca^{2+} permeability could, therefore, not be quantitated. Second, only recently has it become evident that a subpopulation of heavy terminal cisternae-derived skeletal muscle SR vesicles contains a Ca^{2+}-conducting channel which is regulated by external Ca^{2+}, Mg^{2+}, and adenine nucleotides. Third, ryanodine-induced Ca^{2+} efflux is rapid and could not be effectively blocked by inhibitors of the Ca^{2+} release channel. The kinetics of ryanodine-induced $^{45}Ca^{2+}$ efflux could, therefore, not be studied with the use of the relatively slow Millipore filtration method used in this and the previous vesicle studies.

The open SR Ca^{2+} release channel appears to form a rather wide pore which can be traversed not only by Ca^{2+}, but also by Ba^{2+} (11), choline$^+$ (27), and L-glucose (Fig. 5). L-[^{3}H]Glucose efflux was slow enough from ryanodine-treated vesicles to be followed by Millipore filtration. This observation enabled us for the first time to study the mechanism of action of ryanodine which results in an increase in SR Ca^{2+} permeability. We found that the rate of L-[^{3}H]glucose efflux from vesicles treated and not treated with ryanodine was regulated in a similar manner as $^{45}Ca^{2+}$ efflux by external Ca^{2+}, Mg^{2+}, and adenine nucleotide. At low concentrations, ryanodine appears, therefore, to affect the channel in a subtle way. Two possible modes of action are a change in the mechanism of channel activation and inactivation which is not readily detected using the slowly permeating molecule L-glucose or a change in channel structure which prevents its complete closing. In order to distinguish these and other possible mechanisms, a shorter time scale is required for measurements of Ca^{2+} fluxes with ryanodine.

Recent results by Sutko's laboratory[2] support several aspects of the present study. These investigators found also that ryanodine increased the Ca^{2+} permeability of passively loaded junctional heavy skeletal muscle SR vesicles when present in 0.01–10 μM concentrations and decreased the Ca^{2+} permeability when present in concentrations greater than 10 μM. [^{3}H]Ryanodine-binding studies indicated that ryanodine exerted its activating effect by binding to a specific site (10–19 pmol/mg of protein) with a K_D of 150–200 nM. Thus, recent studies by several laboratories suggest that ryanodine may be a useful drug in identifying and characterizing the sarcoplasmic reticulum Ca^{2+} release channel components of skeletal and cardiac muscle.

Acknowledgments—I thank Dr. John L. Sutko for commenting on this manuscript and Julia Henderson and Jeffrey LaDine for excellent technical assistance.

REFERENCES

1. Ebashi, S. (1976) *Annu. Rev. Physiol.* **38,** 293–313
2. Martonosi, A. N., and Beeler, T. J. (1983) in *Handbook of Physiology, Section 10: Skeletal Muscle* (Peachey, L. D., Adrian, R. H., and Geiger, S. R., eds) pp. 417–485, American Physiological Society, Bethesda, MD
3. Inesi, G. (1985) *Annu. Rev. Physiol.* **47,** 573–601
4. Stephenson, E. W. (1981) *Am. J. Physiol.* **240,** C1–C19
5. Ohnishi, S.T. (1981) in *The Mechanism of Gated Calcium Trans-*

[2] F. A. Lattanzio, R. G. Schlatterer, M. Nicar, K. P. Campbell, and J. L. Sutko, (1986) *J. Biol. Chem.*, submitted for publication.

port across Biological Membranes (Ohnishi, S. T., and Endo, M., eds) pp. 275–293, Academic Press, New York
6. Fabiato, A. (1983) *Am. J. Physiol.* **245,** C1–C14
7. Morii, H., and Tonomura, Y. (1983) *J. Biochem. (Tokyo)* **93,** 1271–1285
8. Kirino, Y., Osakabe, M., and Shimizu, H. (1983) *J. Biochem. (Tokyo)* **94,** 1111–1118
9. Nagasaki, K., and Kasai, M. (1983) *J. Biochem. (Tokyo)* **94,** 1101–1109
10. Meissner, G. (1984) *J. Biol. Chem.* **259,** 2365–2374
11. Smith, J. S., Coronado, R., and Meissner, G. (1985) *Nature* **316,** 446–449
12. Ikemoto, N., Antoniu, B., and Meszaros, L. G. (1985) *J. Biol. Chem.* **260,** 14096–14100
13. Meissner, G., Darling, E., and Eveleth, J. (1986) *Biochemistry,* **25,** 236–244
14. Jenden, D. J., and Fairhurst, A. S. (1969) *Pharmacol. Rev.* **21,** 1–25
15. Waterhouse, A. L., Holden, I., and Casida, J. E. (1984) *J. Chem. Soc. Chem. Commun.* 1265–1266
16. Sutko, J. L., Thompson, L. J., Schlatterer, R. G., Lattanzio, F. A., Fairhurst, A. S., Campbell, C., Martin, S. F., Deslongchamps, P., Ruest, L., and Taylor, D. R. (1986) *J. Labeled Compd. Radiopharm.,* **23,** 215–222
17. Pessah, I. N., Waterhouse, A. L., and Casida, J. E. (1985) *Biochem. Biophys. Res. Commun.* **128,** 449–456
18. Fleischer, S., Ogunbunmi, E. M., Dixon, M. C., and Fleer, E. A. M. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82,** 7256–7259
19. Sutko, J. L., Ito, K., and Kenyon, J. L. (1985) *Fed. Proc., Fed. Am. Soc. Exp. Biol.* **44,** 2984–2988
20. Jones, L. R., Besch, H. R., Sutko, J. L., and Willerson, J. T. (1979) *J. Pharmacol. Exp. Ther.* **209,** 48–55
21. Chamberlain, B. K., Levitsky, D. O., and Fleischer, S. (1983) *J. Biol. Chem.* **258,** 6602–6609
22. Feher, J. J., and Lipford, G. B. (1985) *Biochim. Biophys. Acta* **813,** 77–86
23. Fairhurst, A. S., and Hasselbach, W. (1970) *Eur. J. Biochem.* **13,** 504–509
24. Seiler, S., Wegener, A. D., Whang, D. D., Hathaway, D. R., and Jones, L. R. (1984) *J. Biol. Chem.* **259,** 8550–8557
25. Meissner, G. (1985) *Trans. Am. Soc. Neurochem.* **16,** 289
26. Fabiato, A. (1981) *J. Gen. Physiol.* **78,** 457–497
27. Yamamoto, N., and Kasai, M. (1982) *J. Biochem. (Tokyo)* **92,** 485–496